Cyanophycin and arginine metabolism in cyanobacteria

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ABSTRACT

Cyanobacteria are oxygenic photoautotrophs that can utilize inorganic nitrogen salts, atmospheric nitrogen and some amino acids such as arginine as nitrogen source. Under unbalanced growth in the presence of sufficient nitrogen, many cyanobacteria accumulate cyanophycin, a co-polymer of aspartate and arginine that serves as a nitrogen reservoir. Cyanophycin metabolism enzymes include cyanophycin synthetases, cyanophycinase and isoaspartyl dipeptidase, which splits β -aspartyl arginine released from cyanophycin by cyanophycinase into aspartate and arginine. The arginine catabolic pathway of cyanobacteria has been recently elucidated and consists of two bifunctional enzymes, arginine-guanidine removing enzyme (AgrE) and proline oxidase (PutA). This pathway makes available to metabolism the four nitrogen atoms of arginine, three as ammonia and one as glutamate. A variant of the pathway cycles ornithine (an intermediate in the AgrE-catalyzed reactions) back to arginine incorporating aspartate and, hence, recovering its nitrogen atom for metabolism. Many cyanobacteria also make use of this pathway to utilize arginine taken up from the outer medium through a high-affinity ABC transporter. An analysis of co-occurrence in cyanobacteria of the genes encoding enzymes of cyanophycin metabolism, arginine catabolism and the arginine and aspartate transporters indicates a strong correlation between the presence of cyanophycin and the AgrE/PutA pathway.

1. Introduction

Cyanobacteria are a phylogenetically coherent group of bacteria that have the capability to carry out oxygenic photosynthesis [1]. CO₂ fixation is carried out in carboxysomes that are cell inclusions in which carbonic anhydrase and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) are encapsulated in a shell composed of specific proteins [2]. Cyanobacteria can grow photoautotrophically using preferentially ammonium, nitrate or urea as nitrogen source, but many cyanobacteria can fix atmospheric N₂ and, hence, they can grow with air and water containing a few essential mineral salts [3, 4]. Nonetheless, many cyanobacteria are also able to take up some sugars as carbon and energy sources and some amino acids as nitrogen sources [5], which increases the trophic modes of these organisms including mixotrophy –the ability to use simultaneously several redundant growth resources. In spite of a unifying capability for photoautotrophic growth, the cyanobacteria show a remarkable morphological diversity [1]. There are unicellular and multicellular cyanobacteria, and many of the latter can produce differentiated cells specialized for specific functions [6, 7]. The cyanobacteria can also be classified according to the carboxysomes they bear, α or β carboxysomes, which are made of phylogenetically distinct protein components [2, 8]. Notably, whereas most cyanobacteria contain β carboxysomes, the marine unicellular *Synechococcus* and *Prochlorococcus* strains contain α carboxysomes [8].

The enzyme that carries out N₂ fixation, nitrogenase, is extremely sensitive to O₂ posing an especial problem for N₂ fixation in the oxygenic cyanobacteria [9]. Some cyanobacteria such as the marine filamentous organism *Trichodesmium erythraeum* can carry out N₂ fixation in vegetative cells in the light with a possible uncoupling from CO₂ fixation [10, 11], whereas many other cyanobacteria manifestly separate N₂ fixation from photosynthesis. Thus, some unicellular cyanobacteria restrict N₂ fixation to the dark period of diel cycles [12] and some filamentous cyanobacteria restrict N₂ fixation to specialized micro-oxic cells in the filament [6]. This is the case of cyanobacteria that produce cells specialized for N₂ fixation termed heterocysts, which represent 10 % of the cells of the filament, do not perform oxygenic photosynthesis and rely on reduced carbon received from the adjacent vegetative cells [13]. In turn, the heterocysts provide the vegetative cells with fixed nitrogen in the form of amino acids [13]. Under conditions of nitrogen deprivation, some vegetative cells in the filament differentiate into heterocysts in a process that involves a specific program of gene expression [14]. Importantly, heterocystous cyanobacteria are a monophyletic group of organisms [15], implying that the heterocyst evolved only once, likely over 2,100 million years ago [16]. The heterocystous cyanobacteria for which more physiological and molecular data are available belong to the genera *Anabaena* and *Nostoc* (order Nostocales [1]).

A type of cell inclusion present in many cyanobacteria is the cyanophycin granules, which are composed of a material known as cyanophycin granule polypeptide or, simply, cyanophycin. Discovered in, and once thought to be characteristic of cyanobacteria, cyanophycin is now known to be produced by some other bacteria as well [17]. Cyanophycin is a non-ribosomally synthesized polypeptide that consists of a poly- α -aspartic acid peptidic backbone with arginine linked via isopeptide bonds to the β -carboxyl group of each aspartic acid in the backbone (multi-L-arginyl-poly [L-aspartic acid]) [18, 19]. Cyanophycin is a carbon and nitrogen storage polymer that, given its relatively high N to C ratio, can be considered a nitrogen reserve. Excellent reviews covering different aspects of cyanophycin biology are available [17, 20, 21]. Here we shall address the biosynthesis and degradation of cyanophycin and the metabolism of its constituent amino acids in cyanobacteria. We shall also address the capability of cyanobacteria to take up arginine and aspartate from the external medium, as well as a possible correlation between production of cyanophycin and presence of specific arginine utilization pathways.

Two model strains for which much information is available are the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 and the heterocystous cyanobacterium *Anabaena* (also known as *Nostoc*) sp. PCC 7120, which in this article will be referred to as *Synechocystis* and *Anabaena*, respectively. Along the text, we shall identify proteins from these organisms that are related to the topic of this review.

2. Cyanophycin synthesis and degradation

2.1. Genes and enzymes

The cyanophycin granule found in cyanobacteria is an insoluble material that is frequently 25 to 100 kDa in size. As first shown by studies in *Synechocystis*, cyanophycin peptide is elongated by cyanophycin synthetase, the product of the *cphA* gene [22], and degraded by cyanophycinase, the product of the *cphB* gene [23]. Cyanophycin synthetase first adds aspartate to the poly- α -aspartic acid backbone and then adds arginine to the β -carboxyl group of the incorporated aspartate (Fig. 1A). Each of these reactions consume one ATP molecule and is carried out in an independent reaction center in cyanophycin synthetase, which are localized at the N-terminal and C-terminal parts of the enzyme, respectively [24]. Cyanophycinase is a serine-type peptidase that degrades cyanophycin by removing β -aspartyl-arginine dipeptide sequentially [22, 25]. The *cphA* and *cphB* genes are frequently clustered together, with the *cphB* gene located upstream from the *cphA* gene (Fig. 1B). This gene arrangement is conserved in about 90 % of the cyanobacteria that produce cyanophycin (excluding *Pseudanabaena* and *Planktothrix* strains in which this clustering is generally not observed; checked at <https://img.jgi.doe.gov/>). In *Anabaena*, the two genes (termed *cphB1* and *cphA1*, respectively, see next paragraph) are co-transcribed from promoters located

upstream of *cphB1*, and *cphA1* is further transcribed monocistronically from promoters located in the intergenic region [26].

A second *cph* gene cluster, in which the genes are divergent (Fig. 1B), has been described in *Anabaena* [26]. Because in *Anabaena* the genes in the cluster described in the previous paragraph (that is similar to the *Synechocystis* cluster) are responsible for more cyanophycin synthetase and cyanophycinase activities than the genes in the second cluster, the genes in the two clusters have been named *chpA1/cphB1* and *cphA2/cphB2*, respectively [26]. The *cphA2* gene has been found to encode a second type of cyanophycin synthetase that is widespread predominantly in N₂-fixing cyanobacteria [27], in which the divergent arrangement of *cphA2* and *cphB2* is conserved in about 65 % of the genomes containing a *cphA2* gene. CphA2 lacks the C-terminal reaction center found in CphA1 and adds a β -aspartyl-arginine dipeptide to the poly- α -aspartic acid backbone consuming one molecule of ATP per molecule of dipeptide incorporated. In those cyanobacteria that have CphA2, β -aspartyl-arginine can be reincorporated into cyanophycin under conditions that have not yet been defined (Fig. 1A). Whether CphB2 has any specific role in cyanophycin mobilization is unknown.

The full mobilization of cyanophycin involves the cleavage of the β -aspartyl-arginine dipeptide into aspartate and arginine carried out by isoaspartyl dipeptidase, which is related to plant-type asparaginases [28]. Isoaspartyl dipeptidase is composed of two subunits that result from the processing of the polypeptidic product of one gene. To distinguish this gene from the isoaspartyl dipeptidase-encoding gene from bacteria such as *Escherichia coli*, *iadA*, to which the cyanobacterial gene is not related, we shall term *iadC* the gene encoding the cyanobacterial isoaspartyl dipeptidase. In *Anabaena* and *Synechocystis*, *iadC* is open reading frame *all3922* and *sll0422*, respectively. Interestingly, in the streamlined genome of the endosymbiotic cyanobacterium *Richelia intracellularis* HH01 (a symbiont of the diatom *Hemiaulus hauckii* [29]), the *iadC* gene is split into two consecutive genes, *RintHH_8620* and *RintHH_8610*, which is consistent with the importance of producing isoaspartyl dipeptidase to work as two independent polypeptides.

2.2. Physiology

Cyanophycin generally accumulates in the cells under unbalanced growth conditions in the presence of sufficient nitrogen, e.g., when cells are starved for light, sulfur or phosphorus, or when they are incubated at low temperatures or with excess nitrogen-containing compounds (reviewed in [30]). Then, cyanophycin is degraded when balanced growth is resumed [31]. Although cyanophycin is found in many cyanobacteria, it seems not to be required for growth under standard laboratory conditions, since *cphA* mutants from both *Synechocystis* [32] and *Anabaena* spp. [26, 33] are viable. Only under nitrogen limitation and fluctuating light or

light/dark cycles in *Synechocystis* [34], or under diazotrophic and high-light conditions in *Anabaena variabilis* [33], was the growth of a *cphA* or *cphA1* mutant, respectively, impaired.

In N₂-fixing cyanobacteria such as the unicellular strain *Cyanothece* sp. ATCC 51142, which restricts N₂ fixation to the dark period of diel cycles, cyanophycin accumulates in the dark, (when N₂ fixation is operative) and is degraded during photosynthetic growth in the light [32]. Accumulation of recently fixed nitrogen in cyanophycin has also been proposed to be used by *Trichodesmium erythraeum* to temporally uncouple CO₂ fixation and N₂ fixation [11]. A further development in cyanophycin utilization is found in heterocystous cyanobacteria, in which refractile granules that are universally found at the poles of the heterocysts have been identified as cyanophycin [35, 36]. In experiments of fixation of ¹³N-labeled N₂ by *Anabaena cylindrica*, aspartate and arginine were observed just after the primary products of N₂ fixation (ammonia, glutamine and glutamate) were detected [37], which is likely the basis for a rapid incorporation of fixed nitrogen into cyanophycin. Consistently, cyanophycin synthetase activity (measured using aspartic acid or arginine as substrate) is higher in heterocysts than in vegetative cells of *Anabaena* [38], and the *cphA1* gene is expressed in heterocysts as well as in vegetative cells [26]. The cyanophycin granule of the heterocysts appears to be a place of transient accumulation of arginine and aspartate that are to be transferred to the adjacent vegetative cells. Thus, a *cphB1* mutant of *Anabaena* accumulates cyanophycin granules in heterocysts (as well as in vegetative cells) and is impaired in diazotrophic growth (growth rate constant of the mutant is about 64 % that of the wild type [26]). To complete the mobilization of cyanophycin, β-aspartyl-arginine produced by cyanophycinase has to be split into aspartate and arginine but, as shown in *Anabaena*, isoaspartyl dipeptidase accumulates at much higher levels in vegetative cells than in heterocysts, suggesting that the dipeptide is transferred as a nitrogen vehicle to the vegetative cells [39]. Interestingly, an *iadC* mutant of *Anabaena* is impaired in diazotrophic growth to a similar extent as the *cphB1* mutant (growth rate constant of the *iadC* mutant is about 54 % that of the wild type [39]). In summary, cyanophycin is not essential for cyanobacterial growth but appears to provide the cells with a transient storage of aspartate and arginine that improves the metabolic use of nitrogen.

3. Sources of aspartate and arginine

3.1. Cellular synthesis

In experiments of dark fixation of ¹⁴C-labeled CO₂, Melvin Calvin and collaborators found that the earliest fixation product in *Nostoc muscorum* was aspartate [40], which likely resulted from carboxylation of phosphoenolpyruvate (PEP) to produce oxaloacetate and rapid transamination of oxaloacetate to produce aspartate (Fig. 2); more slowly, the second most abundant labeled product was glutamate. *Anabaena* is able to actively take up pyruvate through a tripartite ATP-

independent periplasmic (TRAP) transporter [41], and in *Anabaena* cells incubated with [^{14}C]pyruvate significant accumulation of labeling in aspartate and glutamate has also been observed. This labeling pattern is consistent with carboxylation of pyruvate (via PEP) and (i) quick transamination of oxaloacetate to produce aspartate and (ii) metabolism through the tricarboxylic acid cycle to produce 2-oxoglutarate and, then, glutamate [42] (Fig. 2). A key enzyme encoded in cyanobacterial genomes that is involved in these reactions is PEP carboxylase (All4861 in *Anabaena*; Sll0920 in *Synechocystis*), which in addition to RuBisCo is an important CO_2 fixation enzyme in cyanobacteria. In the case of metabolism of exogenously supplied pyruvate, PEP synthase (at least two orthologues present in *Anabaena*, All0635 and All3146/3147; one in *Synechocystis*, Slr0301) could be involved (Fig. 2). On the other hand, the genome of cyanobacteria such as *Anabaena* or *Synechocystis* bear several genes encoding putative aspartate aminotransferases. Five such genes have been characterized in *Anabaena*, and one of them (*alr4853*) was found to be needed for diazotrophic growth and to encode an enzyme with especially high aminotransferase activity, whereas the other gene products may have redundant activities [43].

The arginine biosynthesis pathway consists of two parts: (i) production of ornithine from glutamate and (ii) transformation of ornithine into arginine. The cyanobacteria produce ornithine from glutamate via the “cyclic pathway of ornithine synthesis” [44], in which glutamate is activated with an acetyl group transferred from acetyl-ornithine by ornithine acetyltransferase [45]. The genes encoding the corresponding enzymes can be detected in sequenced cyanobacterial genomes as exemplified in Figure 3 for *Synechocystis* and *Anabaena*. Additionally, the activity of some of these enzymes has been determined in some cyanobacterial cells, including vegetative cells and heterocysts of *Anabaena* or *Nostoc* spp. (see, e.g., [44, 46, 47]), and two genes (*argC* and *argD*) have been cloned by complementation of arginine auxotrophic mutants of *Anabaena* [48, 49].

N-acetyl-glutamate semialdehyde dehydrogenase is the product of the *argC* gene, and a second gene that encodes a similar enzyme has been found in *Nostoc ellipsosporum* and termed *argL* [50]. The two gene products share low similarity (27% identity between the *Anabaena* proteins), and whereas ArgC is involved in production of arginine for protein synthesis, ArgL appears to be involved in production of arginine that is incorporated into cyanophycin [48, 50]. Although the genome of numerous heterocystous cyanobacteria bear both genes, the presence of the two genes is not a general feature in cyanobacteria (checked at <https://img.jgi.doe.gov/>), in which an *argL*-like gene (generally termed *argC*) is prevalent. The corresponding gene product must be involved in production of arginine for both protein and cyanophycin synthesis. The *argJ* gene encoding ornithine acetyltransferase is also duplicated in *Anabaena* and a few other heterocystous cyanobacteria, but not in *Synechocystis* or the majority of cyanobacteria with sequenced genomes (checked at <https://img.jgi.doe.gov/>). The gene generally found in

cyanobacteria is *argJ1* and, in *Anabaena*, the ArgJ2 protein is 38% identical to ArgJ1. To the best of our knowledge, the corresponding gene products have however not been characterized. Why some heterocystous cyanobacteria have got specialized *argC/argL* genes and duplicated *argJ1/argJ2* genes is unknown, but it would be of interest to investigate whether any of these genes shows cell-specific expression in the *Anabaena* filament.

In [^{14}C]CO₂ fixation experiments with *N. muscorum* (both in the light and darkness), ^{14}C was also found incorporated into citrulline indicating an additional significant site of CO₂ fixation, the synthesis of carbamoyl phosphate [40, 51] carried out by carbamoyl phosphate synthase [46] (Fig. 2), which is the product of the *carA* and *carB* genes (Alr1155 and Alr3809 in *Anabaena*). In the second part of the arginine biosynthetic pathway, ornithine carbamoyltransferase (*argF* gene product) incorporates carbamoyl phosphate into ornithine to produce citrulline [46] (Fig. 3). Mutants of *argF* have been created in *Synechocystis* and *Anabaena*, and both of them are arginine auxotrophs [52, 53]. The final steps of arginine biosynthesis are catalyzed by argininosuccinate synthase that incorporates aspartate with the consumption of ATP, and argininosuccinate lyase, whose activity has been measured in some cyanobacteria (see, e.g., [54]), that produces arginine releasing fumarate (Fig. 3).

In cyanobacteria, the *argB* gene product, N-acetyl-glutamate kinase, is subjected to feed-back inhibition by the final product of the biosynthetic pathway, arginine [44]. The P_{II} protein (*glnB* gene product) transmits information on the C to N balance and energetic (ATP/ADP) status of the cells to several molecular targets [55]. Under nitrogen sufficiency (low C:N ratio) and a high energy status (low ADP levels), non-phosphorylated P_{II} trimer-MgATP complexes tightly bind to N-acetyl-glutamate kinase limiting its sensitivity to arginine and increasing its enzymatic activity [56]. This leads to production of abundant arginine that can be incorporated into cyanophycin [57]. In contrast, under nitrogen deprivation (high C:N ratio), the cells accumulate 2-oxoglutarate that binds to the P_{II} trimer, which becomes phosphorylated preventing its binding to targets. Under these conditions, N-acetyl-glutamate kinase is very sensitive to its allosteric inhibitor arginine, limiting production of arginine that is used for protein synthesis. Thus, N-acetyl-glutamate kinase, which consumes ATP and participates in the first steps of the arginine biosynthetic pathway, is an important target for regulation of arginine biosynthesis with impact on the production of cyanophycin.

3.2. Uptake from the extracellular medium

Many cyanobacteria show an appreciable activity of uptake of amino acids, which is particularly high in the case of arginine transport in *Synechocystis* [58], and arginine has been shown to serve as a nitrogen source in different cyanobacteria [59-61]. The affinity of the cells for arginine is high (K_s around 1 μM or lower) in both *Anabaena* and *Synechocystis* [60, 61]. In contrast, the affinity for aspartate in *Anabaena* is low (K_s , 132 μM [62]), whereas *Synechocystis* shows

negligible uptake of this amino acid [58, 63]. In many cyanobacteria, arginine is taken up by an ATP-binding cassette (ABC) transporter for basic amino acids that consists of two types of subunits: BgtB (Alr3187, Sll1270), which has periplasmic substrate-binding and integral membrane (permease) domains (i.e., it has the primary structure of a fusion of both domains); and BgtA, (Alr4167, Sllr1735) which is a nucleotide-binding domain or ATPase protein [64, 65]. To conform to the standard structure of ABC transporters [66], the functional Bgt transporter is likely composed of two copies of each subunit. Uptake of aspartate in *Anabaena* is mediated by an ABC transporter for acidic (and neutral polar) amino acids known as N-II transporter. This transporter is composed of the products of four genes that likely constitute an operon: *natF* (*alr4164*), encoding a periplasmic substrate-binding protein; *natG* (*alr4165*) and *natH* (*alr4166*), encoding transmembrane proteins; and *bgtA* (*alr4167*), encoding a nucleotide-binding domain [65]. BgtA is shared by the basic and acidic amino acid transporters as evidenced by the fact that its inactivation in *Anabaena* results in transport activities of Arg, Lys and Asp that are about 5 % those of the wild type [65]. Total amino acids in the environment are commonly between 5 and 300 μ M in concentration [67], implying that uptake of aspartate and, especially, arginine from the extracellular medium can be significant in cyanobacteria that express the BgtBA and NatFGH-BgtA transporters. On the other hand, Bgt-mediated uptake of 14 C-labeled arginine has been key in the investigation of arginine utilization that will be discussed below.

4. Arginine catabolism

After mobilization of cyanophycin, aspartate and arginine have to be catabolized to make their nitrogen (and carbon) atoms available for metabolism. Whereas the mode of utilization of aspartate in cyanobacteria has not been specifically investigated, the utilization of arginine has been studied in detail in both *Anabaena* and *Synechocystis*.

Cell suspensions of *Anabaena* or *Synechocystis* supplemented with [14 C]arginine at low μ M concentrations rapidly produce 14 C-labeled proline and glutamate at substantial levels, with some production of 14 C-labeled ornithine and glutamine being also detected [52, 68]. An example of such an experiment is shown in Figure 4. This pattern of labeling is the result of a catabolic pathway that involves two bifunctional enzymes: arginine-guanidine removing enzyme (AgrE; termed ArgZ by Zhang *et al.* [69]) and proline oxidase (PutA) [53], which together produce glutamate from arginine; glutamate can be further used by glutamine synthetase to incorporate ammonia producing glutamine [70]. AgrE is a protein of about 700 amino acid residues (*Anabaena* Alr4995, 703 residues; *Synechocystis* Sll1336, 705 residues) that has an N-terminal domain (residues 10-269 of the *Anabaena* enzyme) with an α/β propeller structure and a C-terminal domain (residues 285-696 of the *Anabaena* enzyme) homologous to an archaeal protein itself with two distinct domains (residues 285-381, similar to a domain in lysine-oxoglutarate

reductase/saccharopine dehydrogenase enzymes; residues 462-696, similar to the NAD/FAD binding domain of deoxyhypusine synthases). The N-terminal domain of AgrE has arginine dihydrolase activity producing ornithine, CO₂ and two ammonium molecules from arginine [53, 69] and the C-terminal domain has ornithine cyclodeaminase activity producing proline and ammonium from ornithine [53] (Fig. 5). Indeed, catabolism of exogenously supplied [¹⁴C]ornithine also produces ¹⁴C-labeled proline and glutamate (Fig. 4a; see also [52, 53]). In the arginine catabolism pathway, proline is further catabolized to glutamate by a conventional PutA (Alr0540, Sll1561), predicted to have proline dehydrogenase and glutamate- γ -semialdehyde dehydrogenase activities, with Δ^1 -pyrroline-5-carboxylate that is spontaneously hydrated to glutamate- γ -semialdehyde as intermediate. Summing up, this pathway makes available to metabolism the four nitrogen atoms of arginine, three as ammonia and one as glutamate (Fig. 5).

The two enzymes of the arginine catabolic pathway, AgrE and PutA, may exert a mechanism of substrate tunneling, in which the product of the first domain of the enzyme (ornithine in AgrE, Δ^1 -pyrroline-5-carboxylate/glutamate- γ -semialdehyde in PutA) is transferred to the second domain avoiding release of the intermediate into the cytoplasm. This phenomenon has been amply investigated in the bacterial PutA protein [71] and is important to avoid futile cycles, i.e., the utilization of ornithine and Δ^1 -pyrroline-5-carboxylate in arginine and proline synthesis, respectively, under conditions that require catabolism of these amino acids. Nonetheless, at least in the case of AgrE, some amount of the intermediate may be released from the enzyme *in vivo*, as some ¹⁴C-labeled ornithine can be detected in physiological assays of catabolism of [¹⁴C]arginine [52, 53]. Part of this ornithine is recycled to produce citrulline and arginine through the second part of the arginine biosynthetic pathway (Figs. 3 and 5). This has been shown in *Synechocystis* by inactivation of *argF* (ornithine carbamoyltransferase), which decreases labeling from [¹⁴C]arginine or [¹⁴C]ornithine into citrulline and increases labeling into proline [52]. Recently, metabolomic analysis of *Synechocystis* upon nitrogen nutrition shifts has also shown the operation of this cycle [69]. Because arginine was thought to be catabolized by an arginase-like enzyme releasing ornithine and urea, the cycle was first referred to as urea cycle [52], but after the identification of the AgrE enzyme with the arginase dihydrolase activity of its N-terminal domain, the cycle has been termed ornithine-ammonia cycle [69]. In this cycle, aspartate is incorporated by the argininosuccinate synthase reaction (Fig. 3), which has the important implication of possibly recovering aspartate (and, hence, one additional nitrogen atom) from cyanophycin (Fig. 5). This can represent a way of utilization of aspartate additional to that represented by aspartate aminotransferases. (Although these enzymes were described above for the biosynthesis of aspartate (Fig. 2), aminotransferases generally catalyze reversible reactions.) How the ornithine-ammonia cycle and the catabolic pathway to glutamate are balanced or whether

one or the other pathway is prevalent under some particular physiological conditions remains to be elucidated.

When an *agrE* mutant of *Anabaena* is incubated with [¹⁴C]arginine, a ¹⁴C-labeled agmatine spot is occasionally observed in thin layer chromatography (see, e.g., Fig. 4b), indicating that the metabolic flux through arginine decarboxylase is increased. Arginine decarboxylase (*speA* gene product, A113401) is the first enzyme in a polyamine biosynthesis pathway that in *Anabaena* produces *sym*-homospermidine [72]. On the other hand, two arginine decarboxylase-encoding genes and their encoded proteins (Slr1312 and Slr0662) have recently been characterized to be involved in spermidine synthesis in *Synechocystis* [73]. The different polyamines accumulated in *Anabaena* and *Synechocystis* reflect the observation that heterocystous cyanobacteria specifically accumulate *sym*-homospermidine that is needed for heterocyst differentiation [72, 74], whereas in *Synechocystis* spermidine content negatively correlates with biofilm formation [73].

5. Co-occurrence of cyanophycin, AgrE/PutA and arginine and aspartate uptake

The scheme in Figure 5 illustrates how the arginine catabolic pathway and the ornithine-ammonia cycle can be involved in the utilization of arginine taken up from the outer medium or released from cyanophycin in cyanobacterial *Anabaena* and *Synechocystis* models. To address how spread these metabolic options are in the phylum Cyanobacteria, we have performed a search for the corresponding genes in a number of sequenced cyanobacterial genomes. As a guide of genomes to be searched, we have used the phylogenetic tree of Shih *et al.* [15]. The data obtained are detailed in supplementary Table 1 and schematically summarized in Figure 6. Cyanophycin biosynthesis and arginine utilization genes are widespread in cyanobacteria other than the marine *Prochlorococcus* and *Synechococcus* (clade C1) and the well-known *Synechococcus elongatus* (clade C2); thus, the α -cyanobacteria (clade C1) specifically lack cyanophycin and the arginine catabolic AgrE/PutA pathway. The presence of cyanophycin metabolism (*cphA*, *cphB*, *iadC*) and arginine catabolic genes (*agrE*, *putA*) strongly correlate as shown in the Venn diagram in Figure 6, and the correlation extends to the deeply divergent *Gloeobacter violaceus* and the recently discovered *Gloeomargarita lithophora* (supplementary Table 1). This correlation has only one notable exception: the marine endosymbiont *Richelia intracellularis* HH01 [29] (see supplementary Table 1), whose genome does not encode the AgrE-PutA pathway. On the other hand, the marine unicellular strain *Synechococcus* sp. PCC 7336 (clade G) bears genes for both the AgrE-PutA pathway and BgtB but not for cyanophycin metabolism enzymes. The peculiarity of *Synechococcus* sp. PCC 7336 is that the evolutionary process of this strain, based on 16S rRNA sequences, differs from that of other *Synechococcus* strains but shares similarity with *Gloeobacter violaceus*, and this strain could therefore represent a new branch in the phylogenetic tree of

cyanobacteria [75]. Overall, available data indicate that the AgrE/PutA pathway provides the normal mechanism for utilization of arginine released from cyanophycin in cyanobacteria.

Among the cyanobacteria that, in the test strains, bear the full set of cyanophycin metabolism and arginine catabolic genes (*cphA*, *cphB*, *iadC*, *agrE*, *putA*), about 43 % also contain the high-affinity basic amino acid transporter Bgt (Fig. 6, Venn diagram). (The presence of the Bgt transporter was searched using the BgtB protein as query; the nucleotide-binding domain, BgtA, was not used because it is also a component of the N-II transporter and, additionally, can be easily confused with the nucleotide-binding domain of other ABC transporters.) The Bgt transporter is widely present in distant cyanobacterial groups, although it is not found in the deeply divergent *Gloeobacter violaceus* and *Gloeobacter kilaueensis* JS1. It appears that, taking advantage of the presence of an efficient arginine catabolic pathway (AgrE/PutA), many cyanobacteria have acquired the efficient Bgt transporter that allows the utilization of arginine that may be found in their natural habitats.

The N-II transporter that is involved in uptake of aspartate was searched in the cyanobacterial genomes using the periplasmic substrate-binding protein NatF and the integral membrane component NatH as queries. The N-II transporter was found to be widespread in cyanobacteria, being present in about 77 % of the test strains and about 76 % of the strains that produce cyanophycin. It was previously known that the N-II transporter is absent from *Synechocystis* [58, 63, 64] and present in *S. elongatus* [76], strains that produce and does not produce cyanophycin, respectively. In spite of the fact that a strict correlation between the presence of the N-II transporter and cyanophycin is not found, the wide distribution of this transporter in cyanobacteria may permit the uptake of aspartate that, when available together with arginine, could be utilized at least in part by the ornithine-ammonia cycle.

6. Concluding remarks

The capability to produce cyanophycin is widespread in β -cyanobacteria, whereas it is absent from α -cyanobacteria. Based on the presence of the nitrogen-rich arginine as one of the cyanophycin constituting amino acids, the cyanophycin granules provide the cells with a dynamic reservoir of nitrogen. This role as a reservoir appears to be especially relevant in N_2 -fixing cyanobacteria, in which recently fixed nitrogen can be transitorily stored in cyanophycin. Additionally, in the case of heterocystous cyanobacteria, cyanophycin plays a central role as a source of β -aspartyl arginine, a dipeptide that serves as a vehicle for the intercellular transfer of nitrogen. The physiology of cyanophycin accumulation and mobilization has been studied for decades, but the pathway for arginine catabolism has been elucidated only recently. The AgrE/PutA pathway makes available to metabolism the four nitrogen atoms of arginine, but a variant of the pathway, the so-called ornithine-ammonia cycle, serves to recover and make

available to metabolism the nitrogen atom of aspartate as well. Future work should clarify the balance between the accumulation and mobilization of cyanophycin and the utilization of arginine and aspartate in cyanobacterial metabolism.

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Author contributions

E.F. conceived the review and wrote the manuscript; S.A. performed database analysis; M.B. performed database analysis, drafted parts of the manuscript and provided experimental data. All authors made manuscript revisions and agree with submission.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

Fig. 1. Cyanophycin synthesis and degradation. (A) Cyanophycin elongation is carried out by the CphA1 type of cyanophycin synthetase, which adds aspartate and arginine to a cyanophycin polypeptide, or by the CphA2 type that adds β -aspartyl-arginine. Cyanophycin degradation is accomplished by the sequential action of cyanophycinase (CphB) and isoaspartyl dipeptidase (IadC). (B) Genomic context of the *cphA* and *cphB* genes in *Synechocystis* and of *cphA1*, *cphB1*, *cphA2* and *cphB2* in *Anabaena* (also known as *Nostoc*) (taken from <https://img.jgi.doe.gov/>). These gene arrangements are highly conserved in cyanobacteria that produce cyanophycin, although *cphA2* and *cphB2* are generally restricted to N_2 -fixing strains.

Fig. 2. Scheme of the central carbon metabolism and synthesis of aspartate in cyanobacteria. Three sites of carbon fixation are indicated (CO_2 or HCO_3^- in red color): the Calvin-Benson-Bassham (CBB) cycle, phosphoenolpyruvate (PEP) carboxylase and carbamoylphosphate synthase. The possible incorporation of exogenously supplied pyruvate by means of PEP synthase is also indicated. The ammonium-assimilating glutamine synthetase-glutamate synthase (GS/GOGAT) cycle is highlighted in blue color in the lower-right part of the scheme ([H], reducing power, normally provided to GOGAT as reduced ferredoxin). The dashed line and arrow indicate the final steps of glycolysis and first steps of the tricarboxylic acid (TCA) cycle: pyruvate kinase, pyruvate dehydrogenase, citrate synthase, aconitase and isocitrate dehydrogenase.

Fig. 3. Arginine biosynthesis pathway in cyanobacteria. Enzyme and gene names, as well as the corresponding *Anabaena* and *Synechocystis* ORFs, are indicated.

Fig. 4. Catabolism of arginine and ornithine in *Anabaena*. (A) Filaments of wild-type *Anabaena* grown under N_2 -fixing conditions (BG11₀) were incubated in the presence of [^{14}C]arginine or [^{14}C]ornithine for the indicated time. (B) Filaments of the *Anabaena agrE* mutant grown in BG11 medium were incubated in the presence of [^{14}C]arginine for the indicated time. In both cases, metabolic products were analyzed by thin layer chromatography as described previously [Burnat & Flores 2014]. In A, note the production of ornithine (Orn), proline and glutamate from arginine and the production of proline, glutamate and arginine from ornithine; the spots of citrulline and glutamine overlap; #8 and 10 are unidentified metabolites. In B, note the production of agmatine (product of arginine decarboxylase) from arginine. White triangles point to the origin of the chromatographies.

Fig. 5. Pathways of utilization of arginine in cyanobacteria. Arginine can be taken up from the outer medium by an ABC transporter for basic amino acids (1) that consists of BgtB (fusion

protein of a periplasmic substrate-binding domain and a transmembrane or permease domain) and BgtA (nucleotide-binding domain or ATPase). Additionally, arginine can be made available to metabolism through degradation of cyanophycin by cyanophycinase (2) and isoaspartyl dipeptidase (3). The arginine catabolism pathway consists of the bifunctional enzymes AgrE (which produces ammonia, CO₂ and, as a reaction intermediate, ornithine) and PutA (which produces glutamate and additionally renders reducing power). A fraction of the ornithine produced in AgrE can be recycled by arginine biosynthetic enzymes ornithine carbamoyltransferase (4), argininosuccinate synthase (5) and argininosuccinate lyase (6), constituting an ornithine-ammonia cycle (OAC). CM, cytoplasmic membrane; [H], reducing power, predictably in the form of FADH₂ (product of the proline dehydrogenase reaction) and NADPH (product of the glutamate- γ -semialdehyde dehydrogenase reaction); P5C/GSA, Δ^1 -pyrroline-5-carboxylate/glutamate- γ -semialdehyde.

Fig. 6. Distribution of cyanophycin biosynthesis and arginine (and aspartate) utilization genes in the cyanobacterial phylum. The phylogenetic scheme is adapted from Shih *et al.* [15]. Representative clades and morphological types of strains in each clade are indicated along with the taxonomic group (I to V) of Rippka *et al.* [3]. Search for the genes in the indicated cyanobacteria was performed by BLAST analysis using the *Anabaena* genes as query, complemented by searches using *Synechocystis* genes. Cyanophycin synthetase is specifically the CphA1-type protein. Ticks indicate the presence of the corresponding gene in 100 % of strains in a clade (highlighted in yellow color), > 90 % (green), > 70 % (blue), > 50 % (magenta), > 20 % (grey). An empty space means absence of the corresponding gene. Detailed information is available in supplementary Table 1. Venn diagram shows the intersection of cyanophycin metabolism (CphA, CphB and IadC proteins) (blue), arginine catabolism (AgrE and PutA proteins) (red), arginine uptake (BgtB) (green) and aspartate uptake (NatFH) (yellow) proteins among the cyanobacterial strains analyzed. *Acaryochloris marina* MBIC11017 was the only strain that presents PutA but not AgrE, and it has been considered not to have the AgrE-PutA pathway in the analysis. Venn diagrams have been calculated and drawn using the tool from the Bioinformatics and Evolutionary Genomics web page (<http://bioinformatics.psb.ugent.be/webtools/Venn/>).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Supplementary Table 1. Cyanophycin metabolism, arginine catabolism, and arginine and aspartate transport genes in cyanobacteria.

The cyanobacteria included in the phylogenetic analysis of Shih *et al.* (2013) were used as test strains. The indicated *Anabaena* genes were used as query in BLAST analysis, which was completed by analysis using as query the corresponding *Synechocystis* genes (except *cphA2*, *natF* and *natH*, not present in this organism). The presence of a gene in a strain is indicated by a check mark (✓). Every strain containing *cphA1* contains *cphB1*, normally in an operon; † indicates that the clustering of the two genes is not conserved. For *cphA2*, ¶ indicates the presence of a *cphB2* gene in the neighbourhood as shown in Fig. 1.

Clade/Strain	Gene							
	<i>cphB1</i> <i>cphA1</i>	<i>cphA2</i>	<i>iadC</i>	<i>agrE</i>	<i>putA</i>	<i>bgtB</i>	<i>natF</i>	<i>natH</i>
Clade A								
<i>Trichodesmium erythraeum</i> IMS101	✓		✓	✓	✓		✓	✓
<i>Lyngbya</i> sp. PCC 8106	✓†		✓	✓	✓		✓	✓
<i>Arthrospira platensis</i> Paraca	✓		✓	✓	✓		✓	✓
<i>Arthrospira platensis</i> NIES-39	✓		✓	✓	✓		✓	✓
<i>Arthrospira</i> sp. PCC 8005	✓		✓	✓	✓		✓	✓
<i>Arthrospira maxima</i> CS-328	✓		✓	✓	✓		✓	✓
<i>Oscillatoria nigro-viridis</i> PCC 7112	✓	✓¶	✓	✓	✓		✓	✓
<i>Microcoleus vaginatus</i> FGP-2	✓	✓¶	✓	✓	✓		✓	✓
<i>Oscillatoria</i> sp. PCC 6506	✓	✓¶	✓	✓	✓		✓	✓
<i>Kamptonema formosum</i> PCC 6407	✓	✓¶	✓	✓	✓		✓	✓
<i>Oscillatoria</i> sp. PCC 10802	✓	✓	✓	✓	✓			
<i>Oscillatoria acuminata</i> PCC 6304	✓		✓	✓	✓		✓	✓
Clade B1								
<i>Fischerella</i> sp. PCC 9605	✓	✓¶	✓	✓	✓	✓		
<i>Fischerella</i> sp. PCC 9431	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Fischerella</i> sp. PCC 9339	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Fischerella</i> sp. JSC-11	✓		✓	✓	✓	✓	✓	✓
<i>Chlorogloeopsis</i> sp. PCC 7702	✓	✓¶	✓	✓	✓	✓		
<i>Mastigocladopsis repens</i> PCC 10914	✓	✓¶	✓	✓	✓	✓		
<i>Calothrix</i> sp. PCC 7103	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Calothrix</i> sp. PCC 6303	✓		✓	✓	✓		✓	✓
<i>Nostoc</i> sp. PCC 7524	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Anabaena</i> sp. PCC 7120	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Anabaena variabilis</i> ATCC 29413	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Nostoc</i> sp. PCC 7107	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Cylindrospermum stagnale</i> PCC 7417	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Anabaena cylindrica</i> PCC 7122	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Anabaena</i> sp. PCC 7108	✓		✓	✓	✓		✓	✓
<i>Raphidiopsis brookii</i> D9	✓		✓	✓	✓		✓	✓
<i>Cylindrospermopsis raciborskii</i> CS-505	✓	✓	✓	✓	✓		✓	✓
' <i>Nostoc azollae</i> ' 0708	✓	✓	✓	✓	✓	✓	✓	✓
<i>Nodularia spumigena</i> CCY9414	✓	✓	✓	✓	✓		✓	✓
<i>Calothrix</i> sp. PCC 7507	✓	✓	✓	✓	✓	✓	✓	✓
<i>Microchaete</i> PCC 7126	✓	✓	✓	✓	✓	✓	✓	✓
<i>Nostoc punctiforme</i> PCC 73102	✓	✓¶	✓	✓	✓	✓		
<i>Scytomena hofmanni</i> UTEX 2349	✓	✓¶	✓	✓	✓			
<i>Rivularia</i> sp. PCC 7116	✓		✓	✓	✓		✓	✓
<i>Synechocystis</i> sp. PCC 7509	✓	✓¶	✓	✓	✓	✓		
<i>Gloeocapsa</i> sp. PCC 7428	✓	✓¶	✓	✓	✓	✓	✓	✓
<i>Chroococcidiopsis thermalis</i> PCC 7203	✓	✓¶	✓	✓	✓	✓	✓	✓
Clade B2								
<i>Halotheca</i> sp. PCC 7418								
<i>Spirulina subsalsa</i> PCC 9445	✓		✓	✓	✓		✓	✓
<i>Spirulina major</i> PCC 6313	✓		✓	✓	✓		✓	✓
<i>Atelocyanobacterium thalassa</i> UCYN-A								
<i>Cyanothece</i> sp. CCY0110	✓	✓¶	✓	✓	✓		✓	✓
<i>Cyanothece</i> sp. ATCC 51472	✓		✓	✓	✓		✓	✓

<i>Cyanothece</i> sp. ATCC 51142	✓		✓	✓	✓		✓	✓
<i>Crocospaera watsonii</i> WH 0003	✓		✓	✓	✓		✓	✓
<i>Crocospaera watsonii</i> WH 8501	✓		✓	✓	✓		✓	✓
<i>Cyanothece</i> sp. PCC 8802	✓		✓	✓	✓		✓	✓
<i>Cyanothece</i> sp. PCC 8801	✓		✓	✓	✓		✓	✓
<i>Synechocystis</i> sp. PCC 6803	✓		✓	✓	✓	✓		
<i>Pleurocapsa</i> sp. PCC 7327	✓		✓	✓	✓		✓	✓
<i>Microcystis aeruginosa</i> NIES-843	✓		✓	✓	✓	✓	✓	✓
<i>Microcystis aeruginosa</i> PCC 7806	✓		✓	✓	✓	✓	✓	✓
<i>Cyanothece</i> sp. PCC 7822	✓	✓	✓	✓	✓	✓	✓	✓
<i>Cyanothece</i> sp. PCC 7424	✓	✓	✓	✓	✓	✓	✓	✓
<i>Prochloron didemni</i> P1								
<i>Gloeocapsa</i> sp. PCC 73106	✓		✓	✓	✓	✓	✓	✓
<i>Synechococcus</i> sp. PCC 7002	✓		✓	✓	✓			
<i>Leptolyngbya</i> sp. PCC 7376	✓		✓	✓	✓		✓	
<i>Cyanobacterium stanieri</i> PCC 7202	✓		✓	✓	✓			
<i>Geminocystis herdmanni</i> PCC 6308	✓		✓	✓	✓	✓		
<i>Cyanobacterium aponinum</i> sp. PCC 10605	✓		✓	✓	✓	✓		
<i>Stanieria cyanosphaera</i> PCC 7437	✓†	✓	✓	✓	✓	✓	✓	✓
<i>Pleurocapsa</i> sp. PCC 7319	✓		✓	✓	✓	✓	✓	✓
<i>Xenococcus</i> sp. PCC 7305	✓		✓	✓	✓		✓	✓
<i>Chroococcidiopsis</i> sp. PCC 6712	✓	✓¶	✓	✓	✓	✓	✓	✓
Clade B3								
<i>Moorea producens</i> 3L	✓		✓	✓	✓		✓	✓
<i>Coleofasciculus chthonoplastes</i> PCC 7420	✓		✓	✓	✓			
<i>Microcoleus</i> sp. PCC 7113	✓	✓¶	✓	✓	✓	✓	✓	✓
Other clade B								
<i>Crinalium epipsammum</i> PCC 9333	✓	✓	✓	✓	✓	✓		✓
<i>Chamaesiphon minutus</i> PCC 6605	✓		✓	✓	✓		✓	✓
<i>Geitlerinema</i> sp. PCC 7105	✓†		✓	✓	✓			
Clade C1								
<i>Synechococcus</i> sp. RCC307								
<i>Prochlorococcus marinus</i> NATL 2A							✓	✓
<i>Prochlorococcus marinus</i> NATL 1A							✓	✓
<i>Prochlorococcus marinus</i> CCMP1986								
<i>Prochlorococcus marinus</i> MIT9515							✓	✓
<i>Prochlorococcus marinus</i> AS9601								
<i>Prochlorococcus marinus</i> MIT9301								
<i>Prochlorococcus marinus</i> MIT9215								
<i>Prochlorococcus marinus</i> MIT9202								
<i>Prochlorococcus marinus</i> MIT9312							✓	✓
<i>Prochlorococcus marinus</i> MIT9211							✓	✓
<i>Prochlorococcus marinus</i> CCMP1375							✓	✓
<i>Prochlorococcus marinus</i> MIT9313							✓	✓
<i>Prochlorococcus marinus</i> MIT9303							✓	✓
<i>Synechococcus</i> sp. WH8102							✓	✓
<i>Synechococcus</i> sp. CC9605							✓	✓
<i>Synechococcus</i> sp. WH8109							✓	✓
<i>Synechococcus</i> sp. BL107							✓	✓
<i>Synechococcus</i> sp. CC9902							✓	✓
<i>Synechococcus</i> sp. WH7805							✓	✓
<i>Synechococcus</i> sp. WH7803							✓	✓
<i>Synechococcus</i> sp. CC9311							✓	✓
<i>Synechococcus</i> sp. WH8016							✓	✓
<i>Synechococcus</i> sp. RS9916							✓	✓
<i>Synechococcus</i> sp. RS9917							✓	✓
<i>Synechococcus</i> sp. WH5701							✓	✓
<i>Synechococcus</i> sp. CB0205							✓	✓
<i>Synechococcus</i> sp. CB0101								
<i>Cyanobium gracile</i> PCC 6307							✓	✓
<i>Cyanobium</i> sp. PCC 7001							✓	✓

Clade C								
<i>Prochlorothrix hollandica</i> PCC 9006	✓		✓	✓	✓		✓	✓
Clade C2								
<i>Synechococcus elongatus</i> PCC 7942							✓	✓
<i>Synechococcus elongatus</i> PCC 6301							✓	✓
Clade C3								
<i>Leptolyngbya</i> sp. PCC 6406							✓	✓
<i>Nodosilinea nodulosa</i> PCC 7104	✓†		✓	✓	✓	✓	✓	✓
<i>Synechococcus</i> sp. PCC 7335	✓		✓	✓	✓		✓	✓
<i>Leptolyngbya</i> sp. PCC 7375	✓		✓	✓	✓		✓	✓
Clade D								
<i>Geitlerinema</i> sp. PCC 7407	✓		✓	✓	✓		✓	✓
<i>Leptolyngbya</i> sp. PCC 6306	✓	✓¶	✓	✓	✓	✓		
Clade E								
<i>Thermosynechococcus elongatus</i> BP-1	✓		✓	✓	✓			
<i>Synechococcus</i> sp. PCC 6312	✓		✓	✓	✓	✓	✓	✓
<i>Cyanothece</i> sp. PCC 7425	✓	✓	✓	✓	✓		✓	✓
<i>Acaryochloris marina</i> MBIC11017					✓		✓	✓
<i>Acaryochloris</i> sp. CCME5410							✓	✓
Clade F								
<i>Synechococcus</i> sp. PCC 7502	✓†		✓	✓	✓		✓	✓
<i>Pseudanabaena</i> sp. PCC 6802	✓†		✓	✓	✓		✓	✓
<i>Pseudanabaena</i> sp. PCC 7429	✓†		✓	✓	✓		✓	✓
<i>Pseudanabaena</i> sp. PCC 7367	✓†		✓	✓	✓	✓	✓	✓
Clade G								
<i>Synechococcus</i> sp. JA-3-3AB							✓	✓
<i>Synechococcus</i> sp. JA-2-3B'a(2-13)	✓†	✓	✓	✓	✓		✓	✓
<i>Synechococcus</i> sp. PCC 7336				✓	✓	✓	✓	✓
Deeply divergent								
<i>Gloeobacter violaceus</i> PCC 7421	✓†		✓	✓	✓			
Recently discovered								
<i>Gloeomargarita lithophora</i> D10	✓		✓	✓	✓			
<i>Gloeobacter kilauensis</i> JS1	✓†		✓	✓	✓			
<i>Richelia intracellularis</i> HH01	✓		✓				✓	✓